I.

Systemic nitroglycerin increases nNOS levels in rat trigeminal nucleus caudalis

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Systemic administration of nitroglycerin, a nitric oxide donor, triggers in migraineurs a delayed attack of unknown mechanisms. Subcutaneous nitroglycerin (10 mg/kg) produced a significant increase of nitric oxide synthase (NOS)- and c-fos-immunoreactive neurons in the cervical part of trigeminal nucleus caudalis in rats after 4 h. This effect was not observed in the thoracic dorsal horn. Similar increase of NOS and c-fos was obtained in the brain stem after a somatic nociceptive stimulus, i.e. on the side of the formalin injection in the lip. Nitric oxide is thus able to increase NOS availability in second order nociceptive trigeminal neurons, which may be relevant for central sensitization and the understanding of its effect in migraine. *NeuroReport* 11:3071–3075 © 2000 Lippincott Williams & Wilkins.

Key words: Migraine; Nitric oxide synthase; Nitroglycerin; Trigeminovascular system

INTRODUCTION

Despite recent advances, the exact pathogenesis of migraine is not fully understood. There is evidence that migraine is a neurovascular disorder with a complex interrelationship between neuronal and vascular mechanisms [1]. One of the human models of migraine is the systemic administration of nitroglycerin (NTG), a nitric oxide (NO) donor. NTG produces a rapid vasodilatation, which is probably responsible for an immediate headache that may occur in healthy subjects, but more often so in migraineurs. After a delay of several hours it also triggers typical attacks without aura in many migraine patients, but not in healthy volunteers [2,3].

NO is formed from L-arginine by nitric oxide synthase (NOS) and as an unconventional transmitter substance it can cross cell membranes rapidly without any specialized release machinery. The synthetizing enzyme has three isoforms: neuronal (nNOS), endothelial (eNOS), and inducible (iNOS). The presence of nNOS in the superficial layers of the dorsal horn of the spinal cord suggests that NO has functions in sensory and pain processing [4]. There is evidence that NO has a key role in hyperalgesia and sensitization of sensory neurons [5]. After s.c. formalin injection in the paw, the number of c-fos- and nNOS-positive neurons increases in the ipsilateral dorsal horn of the appropriate lumbar spinal segment [6,7]. The administration of a NOS inhibitor reduces the c-fos activation, suggesting that NO mediates Fos expression and is involved in control mechanisms affecting nociception [8,9].

Dural mastocytes and trigeminal nerve endings are nNOS positive [10] and the enzyme can also be detected in the caudal trigeminal nucleus [11] and in 5% of the Gasserian ganglion cells [12]. Taken together, this suggests that nNOS is an important actor in trigeminal pain processing.

Using electrical stimulation of trigeminal fibers surrounding the superior sagittal sinus in the cat, which is another experimental model of vascular head pain, activation of second order neurons in the trigeminal nucleus caudalis was reduced after pretreatment with a NOS inhibitor [13]. S.c. NTG injections induce c-fos activation in neurons of the trigeminal system as well as in various other brainstem and forebrain structures [14]. Activation of the latter neurons is thought to be mediated by a primary effect of NO on small caliber nociceptive trigeminal fibers, since it is abolished after capsaicin pretreatment [15].

The cellular and molecular mechanisms of the delayed NTG-triggered migraine attacks are not known. Because of the involvement of the trigeminovascular system in vascular head pain, it has been suggested that this is the most likely target for NO in migraineurs [3]. It has not been determined, however, whether NO would trigger a migraine attack via an effect on the pre- or on the postsynaptic arm of this system. The delay between NTG administration and the attack offers room for a number of central and peripheral actions of NO, including sensitization of peripheral trigeminal afferents comparable to that demonstrated by Strassman *et al.* [16] or central sensitization similar to that shown for second order nociceptive neurons in the spinal cord [8,9]. In any case, changes in NOS activity may play a pivotal role in these processes and in a pilot trial Lassen *et al.* [17] reported that a NOS inhibitor was effective in the treatment of migraine attacks. Increase of nNOS in dural trigeminal nerve endings was

described after NTG administration [18]. NOS activity in the central portions of the trigeminal system has, however, received little attention up to now. The objective of our study was therefore to explore in rats the effect of systemic administration of NTG on nNOS in the trigeminal nucleus caudalis and to compare this effect with that of a somatic nociceptive stimulus in the trigeminal territory, i.e. formalin injections in the upper lip.

MATERIALS AND METHODS

The procedures used in this study followed the guidelines of the International Association for the Study of Pain and the European Communities Council (86/609/EEC). They were approved by the Ethics Committee of the Faculty of Medicine, University of Liège. Thirty adult male Wistar rats (weight 250–350 g) were used. In the first group, eight animals received a single s.c. injection of NTG (prepared from Nitrolingual Pumpspray, Pohl-Boskamp GmbH, Germany) at a dose of 10 mg/kg; eight animals received a s.c. injection of vehicle (gift from Pohl-Boskamp). Four hours later, the rats were deeply anesthetized with sodium pentobarbital (Nembutal, 80 mg/kg) and transcardially perfused with 100 ml physiological saline followed by 500 ml 4% paraformaldehyde in phosphate-buffered saline (PBS). The cervical (C1–C2) and thoracic (Th1) spinal cord was removed. The tissue blocks were postfixed overnight for immunohistochemistry.

The second group of animals consisted of six rats which received NTG ($n=3$) or placebo ($n=3$) according to the same procedures. Four hours later, they were deeply anesthetized and transcardially perfused with 200 ml physiological saline. Cervical (C1–C2) and thoracic (Th1) spinal cords were removed and freezed in liquid nitrogen for western blotting.

Eight animals received s.c. injection of formalin (50 µl, 4%) in the right upper lip. Four hours later the animals were anesthetized and transcardially perfused like the rats in the first group. The brain stem and upper cervical cord were removed, postfixed in the same fixative overnight and processed for nNOS and c-fos immunohistochemistry.

Immunohistochemistry: After cryoprotection (30% sucrose overnight), 30 µm cryostat sections were cut and serially collected in 16 wells containing cold PBS. Each well received sections at a 0.5 mm distance throughout the rostrocaudal extent of the C1–C2 and Th1 segments and the caudal brain stem. After a pretreatment with 0.3% H_2O_2, the free-floating sections were rinsed several times in 0.1 M PBS containing 1% Triton X-100. Then samples from cervical and thoracic spinal cord and the brain stem were kept for 2 nights at 4°C in polyclonal nNOS (Euro-Diagnostica, B 220-1) antibody, at a dilution of 1:20 000. Sections from the cervical spinal cord and the brain stem were incubated with c-fos antibody (Santa-Cruz Biotechnology, sc-52-G) at a dilution of 1:3000 for 3 nights at 4°C. The immunocytochemical reaction was visualized using the avidin–biotin kit (ABC) of Vectastain (Vector Laboratories Inc., PK-6101) and staining with nickel ammonium sulfate-intensified 3',3'-diaminobenzidine. Specificity of the immune reactions was controlled by omitting the primary antiserum.

Western blotting: The dorsal part of the spinal cord segments (C1–C2 and Th1) was homogenized in cold Tris–HCl buffer (50 mM, pH 7.4). Protein concentration was measured according to Bradford using BSA as a standard [19]. Equal amounts of protein samples (20 µg/lane) were separated by standard SDS–PAGE procedures at 200 V for 1 h and transferred to immobilon P membrane (Millipore). Following the transfer and blocking in 5% non-fat dry milk, membranes were incubated with the nNOS antibody (Transduction Laboratories, N31020), diluted to 1:1000. Protein bands were visualized using the ECL Western blotting analysis kit (Amersham). The quantitative analysis was performed using a laser densitometer (Pharmacia LKB).

Statistical analysis: nNOS- and c-fos-positive cells were counted by an observer blinded to the procedures in laminae I–III of the cervical spinal cord, in three different series of sections in each animal. The individual sections in these series were taken at 0.5 mm intervals along the rostrocaudal axis. nNOS-positive neurons were scored if they contained cytoplasmic and dendritic staining and a nucleus. In case of c-fos, the intensively stained nuclei were counted. In the thoracic segment, nNOS-immunoreactive (IR) cells were counted in laminae I–III in ten sections, in each animal. In each experimental group normal distribution was checked by means of the Kolmogorov test. Since the hypothesis of a normal distribution was not rejected, Student's *t*-test was used to determine the significance of differences between the mean values of data groups. In the animals that received s.c. formalin, c-fos- and nNOS-positive cells were also counted on the ipsilateral and contralateral sides of the caudal trigeminal nucleus in the brain stem (three different series per animal) of sections 0.5 mm apart. The difference between the injected and control side for each section was calculated and analyzed with the paired Student's *t*-test. The relative optical densities from western blotting were compared with the Student's *t*-test.

RESULTS

Transverse sections of the cervical spinal cord demonstrated prominent nNOS-IR neurons in the dorsal horn laminae I–III and some cells in the lamina X. In the superficial dorsal horn, small to medium-size neurons (8–15 µm diameter) with few dendrites were most common. The nNOS-IR neurons located around the central canal were multipolar and of medium to large size (15–30 µm). There was no significant difference in the number of NOS-positive cells at different levels of the C1–C2 region. In animals sacrificed four hours after s.c. NTG injection we found a significant increase ($p<0.05$) in the number of nNOS positive neurons as compared to vehicle-treated animals (Fig. 1a,b; Fig. 2a). c-fos-immunopositive cells were identified in dorsal horns; their number was significantly higher in NTG-treated than in placebo-treated animals (Fig. 1c,d; Fig. 2b). By contrast, we found no difference in the number of nNOS-IR neurons between the two groups of animals in the thoracic spinal cord (data not shown).

Western blot analysis of the C1–C2 region confirmed the results obtained by immunohistochemistry. We could identify a band at 155 kDa characteristic for the nNOS protein.

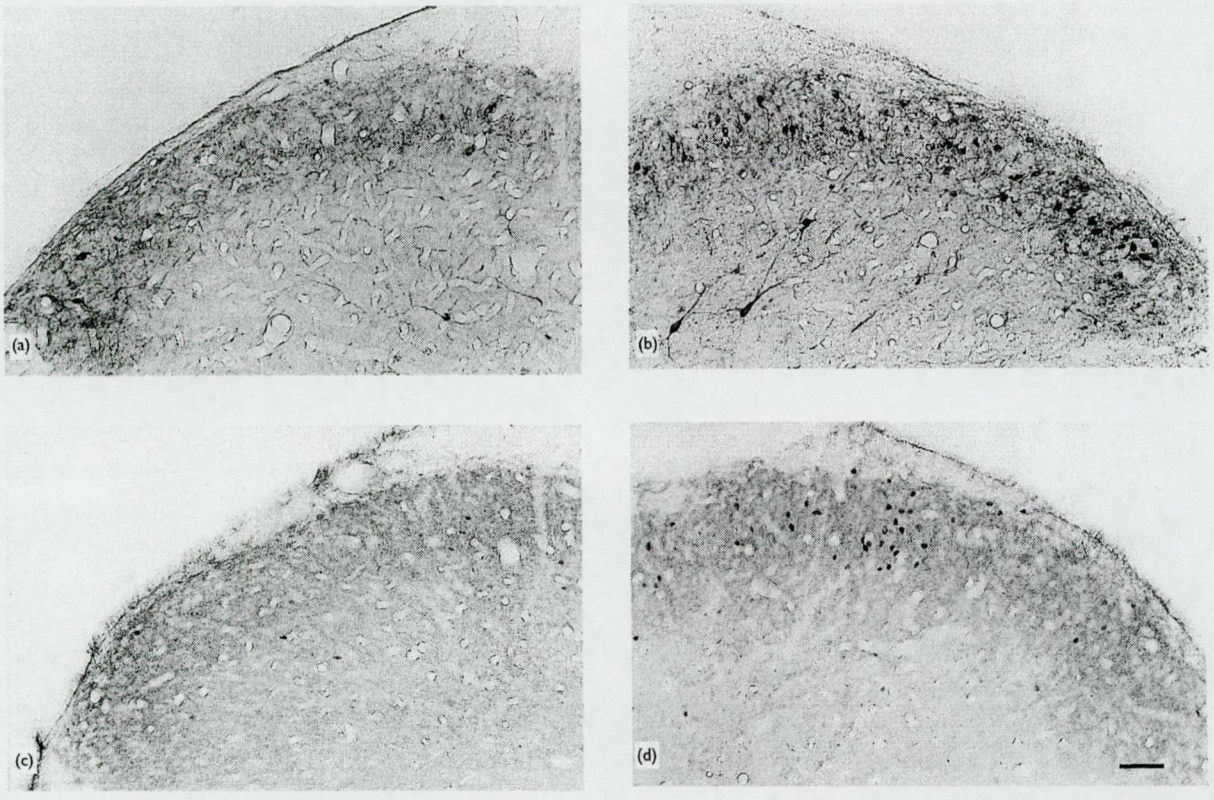

Fig. 1. nNOS (**a**,**b**) and c-fos (**c**,**d**) immunoreactivity in transverse sections of the upper cervical dorsal horn. The number of immunoreactive cells is increased 4 h after s.c. NTG (10 mg/kg) (b,d), compared to placebo (a,c). Scale bar = 50 μm.

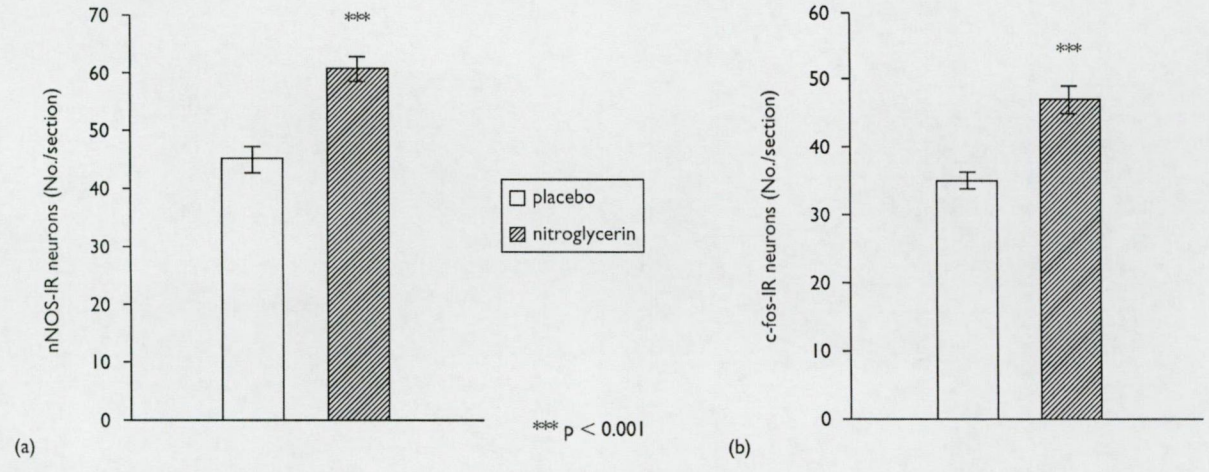

Fig. 2. Histogram illustrating the significant difference between NTG and placebo-treated rats in number of nNOS- (**a**) and c-fos- (**b**) imunoreactive neurons per 30 μm transverse sections in the cervical dorsal horn (mean ± s.e.m.; $n = 8$).

In animals which had received NTG 4 h previously the density of the nNOS protein band was higher in C1–C2 segments, but not in the Th1 segment (Fig. 3). Densitometric analyses confirmed that the nNOS band on Western blots was significantly enhanced after NTG administration in cervical (control: 103.7 ± 2, NTG-treated: 149.7 ± 5.8, $p < 0.01$), but not in thoracic segments (control: 107 ± 4.3, NTG-treated: 116.7 ± 4.4, n.s.).

In animals which were given an s.c. formalin injection in the lip, the number of both nNOS- (Fig. 4a) and c-fos- (Fig.

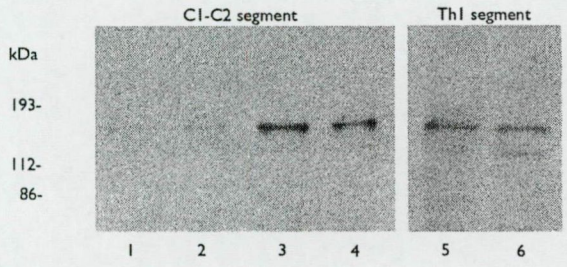

Fig. 3. Western blotting of nNOS in the cervical and thoracic regions of rat spinal cord. Lanes 1, 2 and lane 5 after placebo, lanes 3, 4 and lane 6 after NTG administration. After s.c. NTG (10 mg/kg) treatment nNOS levels (155 kDa) are increased in the C1–C2 segment, but not in the Th1 segment.

4b) positive cells in the caudal trigeminal nucleus increased ipsilaterally to the injection in the appropriate segment of the cervico-medullary junction.

DISCUSSION

Our data demonstrate that systemic NTG administration increases significantly nNOS immunoreactivity in the superficial dorsal horn of the upper cervical spinal cord, in addition to the effect already described for c-fos immunoreactivity [14]. To understand the mechanisms responsible for the nNOS increase and its possible relevance for migraine it is important to note that NTG-induced c-fos- and nNOS-positive neurons appear in a comparable anatomic territory, which receives C and Aδ afferents from peripheral somatic trigeminal territories and meningeal vessels [20]. This suggests that both immunoreactivities are interdependent and related to activation of second order nociceptive neurons and interneurons. Such a relationship was demonstrated in the spinal cord after intradermal capsaicin injections [9] and more recently in trigeminal nucleus caudalis after formalin injections into the face, which resulted in co-localization of c-fos and nNOS in 14% of neurons [21].

Increased nNOS immunoreactivity may be due to increased synthesis or to reduced utilization and cytoplasmic accumulation. Because of the concomitant c-fos activation and the fact that NTG or nociceptive stimuli cause activation of second order neurons, it is more likely that increased synthesis is causing this phenomenon.

The nNOS increase in second order trigeminal nociceptors could be due to a direct effect of NO on these neurons, to a modulation by NO of descending brain stem inputs or to an indirect activation via NO stimulation of peripheral nociceptive afferents. Since NTG produced no detectable changes in nNOS content of the thoracic dorsal horn, a direct effect of NO on second order sensory neurons containing nNOS is most unlikely. There is some evidence that local microinjections of NO donors in the rat are able to inhibit periaqueductal gray (PAG) matter neurons [22]. The ventrolateral part of PAG can inhibit neurons in trigeminal nucleus caudalis, including those that receive afferents from intracranial vessels [23]. Although the studies on local injections of NO donors were performed on the dorsolateral, i.e. the autonomic, portion of PAG, we cannot rule out that the nNOS activation in trigeminal nucleus caudalis is secondary to disinhibition because of reduced descending inputs from PAG neurons. The most likely explanation for the increased nNOS immunoreactivity, however, is the secondary activation of second order nociceptive neurons and/or interneurons because of excitation of their peripheral afferents. NTG is indeed able to activate meningeal nociceptive fibers [18] and we have confirmed in the present paper that a somatic nociceptive stimulus (s.c. formalin) is able to activate both c-fos and nNOS in the trigeminal system. It has been shown, moreover, that c-fos activation in trigeminal nucleus caudalis neurons by s.c. NTG is reduced after the destruction of unmyelinated fibers by capsaicin [15] and that c-fos activa-

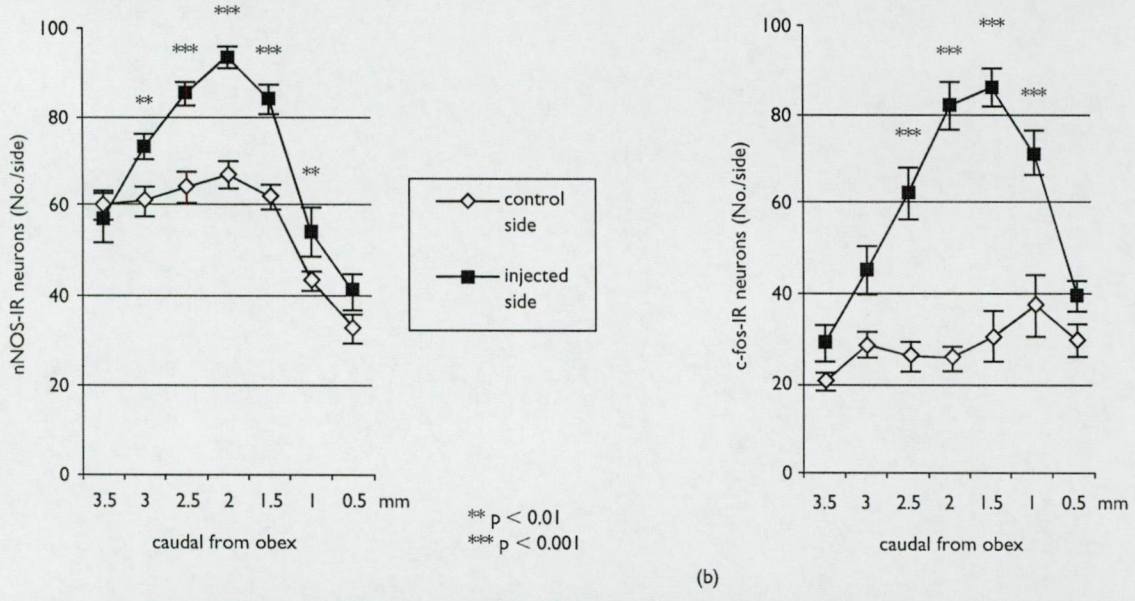

Fig. 4. Histogram showing the number of nNOS (**a**) and c-fos (**b**) immunoreactive neurons in the caudal trigeminal nucleus in 30 μm transverse sections at increasing caudal distances from obex, ipsi- and contralaterally to an s.c. formalin injection in the lip (mean ± s.e.m.; $n = 8$).

tion by NO donors in the spinal cord is abolished by pretreatment with a NOS inhibitor [9].

Our findings could be relevant for the pathophysiology of the trigeminovascular system and thus for migraine pathogenesis and its NO hypothesis. Increased nNOS activity in superficial trigeminal nucleus caudalis suggests that the local availability of NO is enhanced. This may be responsible for a central sensitization, possibly via activation of c-fos which can induce a number of excitatory or inhibitory transmitters related to pain mechanisms; such a relationship between NO and c-fos was recently demonstrated in the spinal cord after a peripheral nociceptive stimulus [5,9]. The effects of NO on nNOS activity in second order nociceptive neurons appear to be specific to the trigeminal system, as we did not observe them in the thoracic spinal cord. The neurobiology of trigeminal and peripheral nociception may thus be different. Whether this difference could be related to the different presynaptic receptor population (e.g. 5HT 1 B/D receptors) on nociceptive afferents, which is underscored by the selective efficacy of triptans in migrainous headache [24], or to other factors remains to be proven. In any case the data presented here suggest that NO donors are able to initiate a feedback process in which their direct effect on nociceptive trigeminovascular afferents may be amplified and prolonged by the nNOS activation in trigeminal nucleus caudalis. Such a process might be important for central sensitization and, possibly, for migraine headache. It may be related to the clinical signs of central trigeminal nociceptor sensitization which were recently shown in migraine patients [25]. It might also explain why NTG causes an attack in migraineurs only after a delay of several hours, which corresponds roughly to the time it takes to induce the increased nNOS activity in the rat.

If increased nNOS activity in the trigeminal nucleus caudalis is pivotal in NTG-induced, and possibly spontaneous attacks of migraine, it can be used as an experimental model for the study of migraine pathogenesis and of potential anti-migraine drugs.

CONCLUSION

We have demonstrated that systemic NTG administration in rats causes a significant increase in nNOS and c-fos immunoreactivity in the cervical portion of the nucleus trigeminalis caudalis, which receives most of the nociceptive afferents from the dural vasculature. Western blotting confirmed this nNOS increase. The effect of NTG was not found in the thoracic spinal cord, but it was similar to a somatic nociceptive stimulus in the trigeminal territory, i.e. lip injection of formalin.

Our results suggest that NO may induce a self-amplifying process at the central projection site of trigeminovascular afferents, which may play a role in sensitization and the delayed occurrence of migraine attacks triggered by systemic administration of NTG, and possibly of spontaneous attacks. The NO-induced increase of nNOS in second order trigeminal neurons could be an interesting model to study established and potential anti-migraine drugs.

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II.

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Effect of systemic nitroglycerin on CGRP and 5-HT afferents to rat caudal spinal trigeminal nucleus and its modulation by estrogen

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Keywords: 17 β-estradiol, 5-HT, caudal spinal trigeminal nucleus, CGRP, nitroglycerin

Abstract

Systemic administration of nitroglycerin, a nitric oxide donor, triggers in migraine patients a delayed attack of unknown mechanism. After puberty migraine is more prevalent in women. Attacks can be triggered by abrupt falls in plasma estrogen levels, which accounts in part for sexual dimorphism, but lacks an established neurobiological explanation. We studied the effect of nitroglycerin on the innervated area of calcitonin gene-related peptide (CGRP) and serotonin-immunoreactive afferents to the superficial laminae of the spinal portion of trigeminal nucleus caudalis, and its modulation by estrogen. In male rats, nitroglycerin produced after 4 h a significant decrease of the area innervated by CGRP-immunoreactive afferents and an increase of that covered by serotonin-immunoreactive fibres. These effects were not observed in the superficial laminae of thoracic dorsal horns. The effect of nitroglycerin was similar in ovariectomized females. In estradiol-treated ovariectomized females the area in the spinal portion of trigeminal nucleus caudalis laminae I–II covered by CGRP-immunoreactive fibres was lower and that of serotonin-immunoreactive fibres was higher than in males and for both transmitters not significantly changed after nitroglycerin. The bouton size of CGRP profiles was smaller in estradiol-treated ovariectomized females, whereas after nitroglycerin it decreased significantly but only in males and ovariectomized females. Nitroglycerin, i.e. nitric oxide, is thus able to differentially influence afferent fibres in the superficial laminae of rat spinal trigeminal nucleus caudalis. Estradiol modulates the basal expression of these transmitters and blocks the nitroglycerin effect. These data may contribute to understanding the mechanisms by which estrogens influence migraine severity and the triggering of attacks by nitric oxide.

Introduction

Systemic administration of nitroglycerin (NTG) triggers a typical attack without aura after a delay of several hours in migraineurs, but not in healthy volunteers (Sicuteri *et al.*, 1987; Olesen *et al.*, 1993). The precise mechanisms by which NTG, and thus nitric oxide (NO), triggers migraine are not known, but the delay of the effect does not favour a pure vascular action.

Gonadal steroids, in particular estradiol, modulate the clinical expression of migraine. After puberty women are three times more affected than men. Abrupt falls in estrogen plasma levels can trigger the attacks, e.g. in the premenstrual phase (Somerville, 1975) and they may disappear during pregnancy or after menopause, when the plasma level of estrogen is stable (Marcus, 1995; Silberstein & Merriam, 2000). The neurobiological mechanisms which underlie these modulatory effects of estrogen on migraine remain speculative.

In rats systemic NTG activates a widespread set of neurons including second-order nociceptors in the caudal spinal trigeminal nucleus (Tassorelli & Joseph, 1995), where most trigeminovascular nociceptive afferents project (Goadsby & Hoskin, 1997). NTG increases the expression of nitric oxide synthase (NOS) in these neurons (Pardutz *et al.*, 2000), suggesting that NO stimulates trigeminal Aδ and C afferents and via second-order nociceptors induces a self-amplifying process possibly responsible for central sensitization.

Calcitonin gene-related peptide (CGRP), a key transmitter in primary nociceptive afferents, can be released in animals by NO-mediated mechanisms (Garry *et al.*, 2000) and increases in jugular blood during migraine attacks (Goadsby *et al.*, 1990). After electrical stimulation of the Gasserian ganglion in rats, an animal model for migraine producing meningeal plasma extravasation (Buzzi & Moskowitz, 1992), CGRP-immunoreactive (Ir) fibres in dura mater undergo morphological changes suggestive of transmitter release (Knyihar-Csillik *et al.*, 1995, 2000). These CGRP changes in migraineurs and in rats are both reversed, viz. prevented by serotonin $(5\text{-HT})_{1B/D}$ agonists, the most effective acute antimigraine drugs. Serotonergic mechanisms are pivotal in the control of nociception (Roberts, 1984; Fasmer *et al.*, 1985) and in migraine pathogenesis (Sicuteri, 1972; Ferrari *et al.*, 1989).

There is experimental evidence for an effect of estrogen on both CGRP and 5-HT neurotransmission. Estradiol reduces the CGRP

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content in cervical spinal cord (Moussaoul *et al.*, 1996) and the number of CGRP-Ir neurons in sensory ganglia (Yang *et al.*, 1998). It also increases in the raphe nuclei the expression of tryptophan hydroxylase, the rate-limiting enzyme of 5-HT synthesis, and of its mRNA (Pecins-Thompson *et al.*, 1996; Lu *et al.*, 1999; Bethea *et al.*, 2000), whilst it decreases that of the serotonin re-uptake transporter (Pecins-Thompson *et al.*, 1998; Rehavi *et al.*, 1998).

As CGRP and 5-HT are involved in migraine pathogenesis and both are influenced by estrogen, they could be pivotal for a better understanding of the hormonal influences and other trigger factors in this disorder. We therefore examined, in rats, the effect of NTG administration on the innervation area of 5-HT and CGRP afferents to the superficial laminae of spinal C1–C2 portion of trigeminal nucleus caudalis (sTNC) and whether this effect, as well as the baseline expression of these transmitters, can be modulated by estradiol.

Materials and methods

Animals

The procedures in this study followed the guidelines of the International Association for the study of Pain and the European Communities Council (86/609/ECC). They were approved by the Ethics Comittee of the Faculty of Medicine, University of Liège. Sixteen male and 32 female Wistar rats (250–350 g) were used. The animals were raised and maintained in standard laboratory conditions with tap water and regular rat chow available *ad libitum* on a 12-h dark : 12-h light cycle.

At the age of 2 months, the female animals ($n = 32$) were ovariectomized under Nembutal° anaesthesia and half of them had a 5-mm-long Silastic capsule (3.18 mm OD and 1.57 mm ID; Dow Corning, Midland, MI, USA) filled with a 1 : 1 mixture of cholesterol (Sigma Chemical Co., St. Louis, MO, USA) and 17β-estradiol (Fluka, Buchs, Switzerland) inserted subcutaneously in the interscapular region. The capsules maintain estradiol plasma levels in a range that is typical of those found in female rats at early proestrus. (Smith *et al.*, 1977).

Nitroglycerin administration

At the age of 3 months, half of the animals in all three groups [eight males, eight ovariectomized females (ovx), eight ovariectomized females treated with estradiol (ovx + E_2)] received a subcutaneous injection of NTG (prepared from Nitrolingual° spray, Pohl-Boskamp GmbH, Germany) at a dose of 10 mg/kg. The other half received an injection of the vehicle (gift from Pohl-Boskamp GmbH) in the same location. Four hours after NTG or vehicle injections, the rats were deeply anaesthetized with pentobarbital (Nembutal°80 mg/kg; Sanofi-Synthélabo, Paris, France) and transcardially perfused with 100 mL physiological saline followed by 500 mL 4% paraformaldehyde in phosphate-buffered saline. The cervical (C1–C2) spinal cords from all animals, as well as the thoracic (Th1) cord from males, were removed and postfixed overnight for immunohistochemistry.

Immunohistochemistry

After cryoprotection (30% sucrose overnight), 30-μm-thick transverse cryostat sections were serially cut and collected in 16 wells containing cold phosphate-buffered saline. Each well received sections separated by 0.5 mm from the entire rostro-caudal extent of the C1–C2 and the Th1 spinal segments. After pretreatment with 0.3% H_2O_2, the free-floating sections were rinsed several times in 0.1 M phosphate-buffered saline containing 1% Triton X-100 and then kept for 2 nights at 4 °C in polyclonal anti-CGRP (Amersham Biosciences AB, Uppsala, Sweden; RPN. 1842) or anti5-HT (DiaSorin Inc., Stillwater, MN, USA, 20080) primary antisera at respective dilutions of 1 : 20 000 and 1 : 200 000. The immunocytochemical reaction was visualized using the Vectastain° (Vector Laboratories Inc., Burlingame, CA, USA; PK-6101) avidin–biotin kit (ABC) with nickel–ammonium–sulphate-intensified 3′,3′-diaminobenzidine (Sigma). Specificity of the immune reactions was controlled by omitting the primary antisera.

Data analysis

The area covered by CGRP- and 5-HT-Ir fibres in laminae I–II of the cervical and thoracic dorsal horns was determined by video imaging using an Image Pro Plus 4.0 image analysis software (Media Cybernetics, Silver Spring, MD, USA). Stained sections were examined under bright field with an Olympus microscope and a 10× objective. Images were recorded with a SONY 950-P CCD camera (Sony Corp., Japan) and transmitted to the frame grabber (Flashpoint 128; Integral Technologies, Inc., Indianapolis, IN, USA) which converts the image into a digital matrix of 1600 × 1200 pixels. After image acquisition, a threshold grey level was established in order to detect Ir fibres in the digitized microscopic image, the so-called discrimination step. To avoid the subjective bias of manual thresholding, the threshold was determined on the basis of the density histogram displayed by the program. It was set on the point where the flat part of the histogram (pixels with high densities) started to rise steeply. The program expressed the area innervated by the IR fibres as number of pixels having densities above the threshold. For the calibration we measured known areas of different shapes. Using sections from the thoracic spinal cord processed in parallel we tested reproducibility of measurements in homologous areas of the dorsal horns. Measurements were taken in a blinded fashion from at least 16 sections for each staining in each animal group, and averaged.

The size of immunoreactive boutons was measured by the same digital system using a 40× objective. At this high magnification different optical planes of the same section could be examined. For the determination of the cross-sectional areas we selected boutons which were in focus and were recognized and measured by the program as single objects. In each experimental group 450–500 boutons were analysed.

Generalized linear models were used to compare the data in function of group and treatment. Within each of the three groups of animals (males, ovx and ovx + E_2), differences between NTG and vehicle treatment were analysed with Student's *t*-test. The analyses were performed by the software SAS (Version 6.12 for Windows; SAS Institute, Cary, NC, USA). Significance level was set at $P < 0.05$.

Results

On transverse sections of the C1–C2 spinal segments there were abundant CGRP-Ir fibres in the superficial layers of the caudal spinal trigeminal nucleus. The area covered by these fibres was not significantly different between the various rostro-caudal levels, nor between sides of the C1–C2 segments.

As far as the total area covered by CGRP-Ir in the superficial laminae I–II of sTNC after vehicle injection was concerned, we found no significant difference between male rats and ovariectomized females. By contrast, the CGRP-innervated area in the estradiol-treated ovariectomized group was significantly smaller than in any of the two other groups (Fig. 1A, C and E).

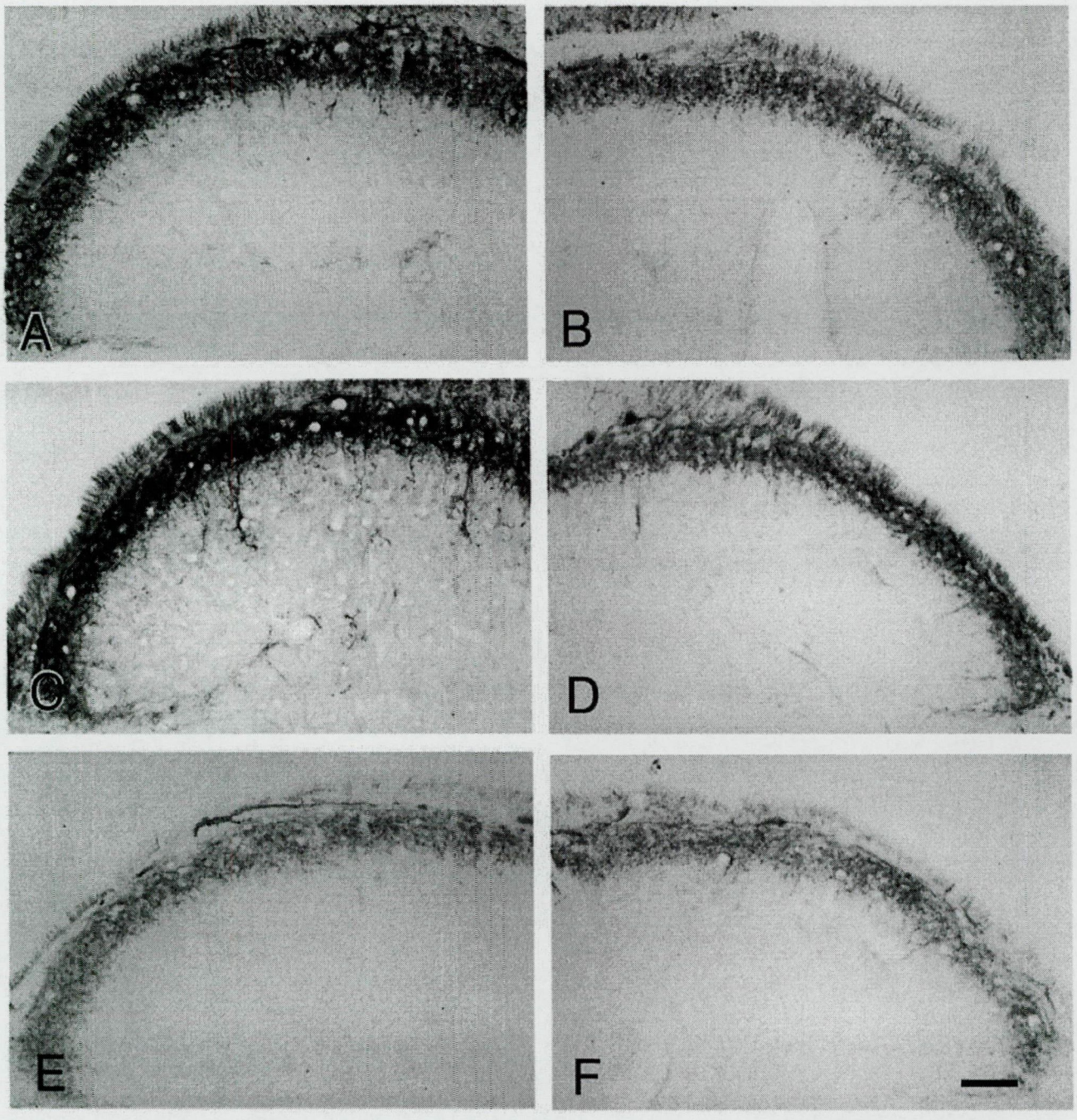

FIG. 1. CGRP immunoreactivity on transverse sections of the upper cervical spinal cord in (A and B) males, (C and D) ovx and (E and F) ovx + E_2 females. (B, D and F) NTG administration compared to (A, C and E) vehicle reduces the immunoreactivity in (B and D) males and ovx females but not in (F) ovx + E_2 rats where the baseline immunoreactivity is lower. Scale bar, 50 μm.

Four hours after subcutaneous NTG administration there was a significant decrease of the area covered by CGRP-IR fibres in males (Fig. 1A and B) and in ovariectomized females (Fig. 1C and D) compared to vehicle injections, but no change was found in estradiol-treated ovariectomized rats (Fig. 1E and F).

At the Th1 level in male rats, where the superficial dorsal horn area innervated by CGRP fibres is much smaller, there was no significant difference between NTG- and vehicle-injected animals (data not illustrated).

These results of the CGRP innervation are synoptically presented in the histogram of Fig. 2 and in Table 1.

At higher magnifications the size of the CGRP-Ir boutons in laminae I–II of sTNC was not different between males and ovariectomized females but was significantly lower in the estradiol treated group. After NTG injections, the bouton size decreased significantly in males (Fig. 3 and Table 2) and ovariectomized animals, but not in the ovariectomized + estradiol treated rats. We found no changes in bouton sizes at the Th1 level (Fig. 4).

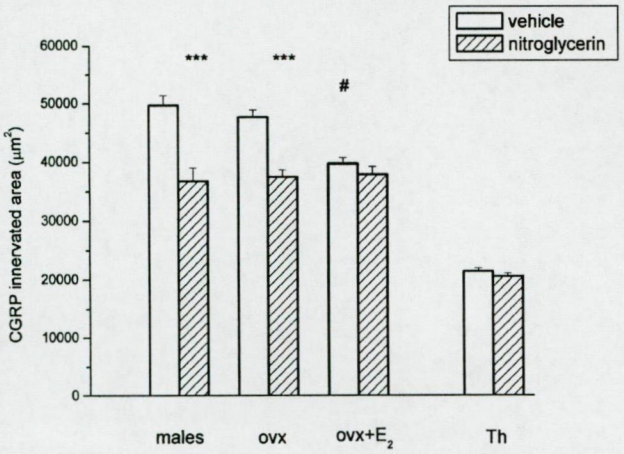

FIG. 2. Histogram showing the area in μm^2 covered by CGRP-Ir fibres in superficial laminae I–II of sTNC at C1–C2 in the three animal groups and of segment Th1 (th) in males 4 h after subcutaneous injection of vehicle (light bars) or NTG (hatched bars) (mean + SEM, $n = 8$ per group). In males and ovx animals there is a significant decrease in the area innervated by CGRP-Ir fibres ($^{***}P < 0.001$). The level after vehicle injection is significantly lower in ovx + E_2 animals as compared to the male or ovx rats ($^{\#}P < 0.05$).

TABLE 1. Areas innervated by CGRP and 5-HT fibres in the superficial laminae of sTNC and the thoracic (Th) dorsal horns

	CGRP		5-HT	
	Vehicle	Nitroglycerin	Vehicle	Nitroglycerin
Males	4.97 ± 0.16	3.67 ± 0.23***	3.16 ± 0.12	4.07 ± 0.06***
Ovx	4.76 ± 0.12	3.75 ± 0.13***	3.17 ± 0.17	4.2 ± 0.16***
Ovx + E2	3.98 ± 0.1†	3.79 ± 0.14	4.08 ± 0.17†††	4.37 ± 0.14
Th	2.15 ± 0.05	2.06 ± 0.06	1.69 ± 0.06	1.67 ± 0.07

The values are expressed as means ± SEM in 10^4 μm^2. There are significant differences ($^{***}P < 0.001$) between vehicle and nitroglycerin in the Male and Ovx group as well as after vehicle between Males and Ovx + E2 animals ($^{\dagger}P < 0.05$, $^{\dagger\dagger\dagger}P < 0.001$).

Serotonergic fibres project to the entire grey matter of the spinal cord, but the densest innervation by 5-HT-Ir fibres is found in superficial laminae I–II of the dorsal horn. This is also the case at the level of sTNC. Similarly to the results obtained with CGRP, the area in sTNC laminae I–II innervated by 5-HT-Ir fibres was not significantly different in male rats compared to ovariectomized females. By contrast with CGRP-fibres, however, the 5-HT-Ir area was significantly greater in the estradiol-treated ovariectomized animals than in the two other groups (Fig. 5A, C and E).

Whilst the area occupied by 5-HT-Ir fibres in the superficial dorsal laminae at C1–C2 segmental levels significantly increased after NTG injections in male (Fig. 5A and B) and ovariectomized rats (Fig. 5C and D), these injections had no effect on the 5-HT innervation in estradiol-treated ovariectomized animals (Fig. 5E and F).

At segmental level Th1, the area covered by 5-HT-Ir fibres in laminae I–II of the dorsal horns was much smaller (not illustrated) than the one measured at C1–C2 and it remained unchanged after NTG administration (see Fig. 6).

The results for 5-HT-Ir areas are summarized in the histogram of Fig. 6 and in Table 1. At higher magnification there was no

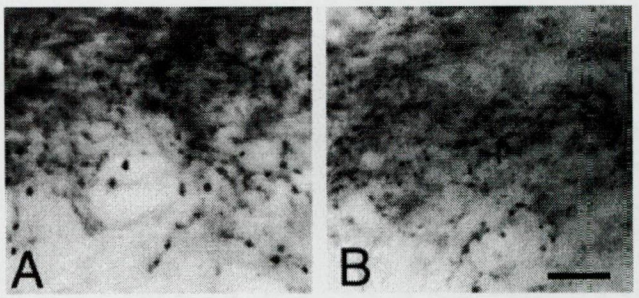

FIG. 3. High magnification photomicrograph of CGRP-immunoreactive boutons in laminae I–II of sTNC of (A) vehicle- and (B) nitroglycerin-treated male rats. After NTG administration the bouton size is smaller. Scale bar, 10 µm.

TABLE 2. Size of CGRP- and 5-HT immunoreactive boutons expressed as the cross-sectional area in μm^2 (mean ± SEM)

	CGRP		5-HT	
	Vehicle	Nitroglycerin	Vehicle	Nitroglycerin
Males	0.64 ± 0.03	0.49 ± 0.01***	0.56 ± 0.04	0.61 ± 0.05
Ovx	0.65 ± 0.02	0.48 ± 0.01***	0.56 ± 0.03	0.59 ± 0.03
Ovx + E2	0.51 ± 0.02 †	0.51 ± 0.01	0.58 ± 0.03	0.59 ± 0.04
Th	0.64 ± 0.02	0.63 ± 0.02	0.6 ± 0.03	0.61 ± 0.04

There is a significant reduction in the size of CGRP-Ir boutons ($^{***}P < 0.001$) between vehicle- and nitroglycerin-treated Male and Ovx animals. After vehicle administration the boutons are significantly smaller ($^{\dagger}P < 0.05$) in Ovx + E2 rats than in the males.

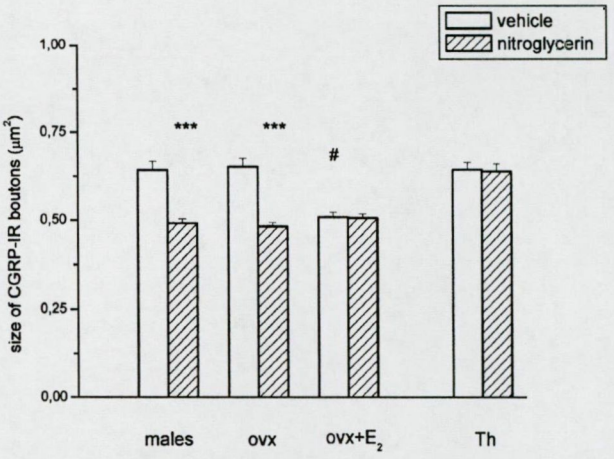

FIG. 4. Histogram showing the CGRP-Ir bouton size in superficial laminae I–II of sTNC at C1–C2 in the three groups of animals and in segment Th1 (th) of males 4 h after subcutaneous injection of vehicle (light bars) or NTG (hatched bars) (mean + SEM, $n = 8$ per group). In males and ovx animals there is a significant decrease in the bouton size of CGRP-Ir fibres ($^{***}P < 0.001$). The bouton size after vehicle injection is significantly lower in ovx + E_2 animals than in the male or ovx rats ($^{\#}P < 0.05$).

detectable size difference of serotoninergic boutons in the superficial layers of sTNC between animal groups nor before nor after NTG administration (Table 2). The numerical data for CGRP- and 5-HT-Ir area and bouton size are summarized in Tables 1 and 2, respectively.

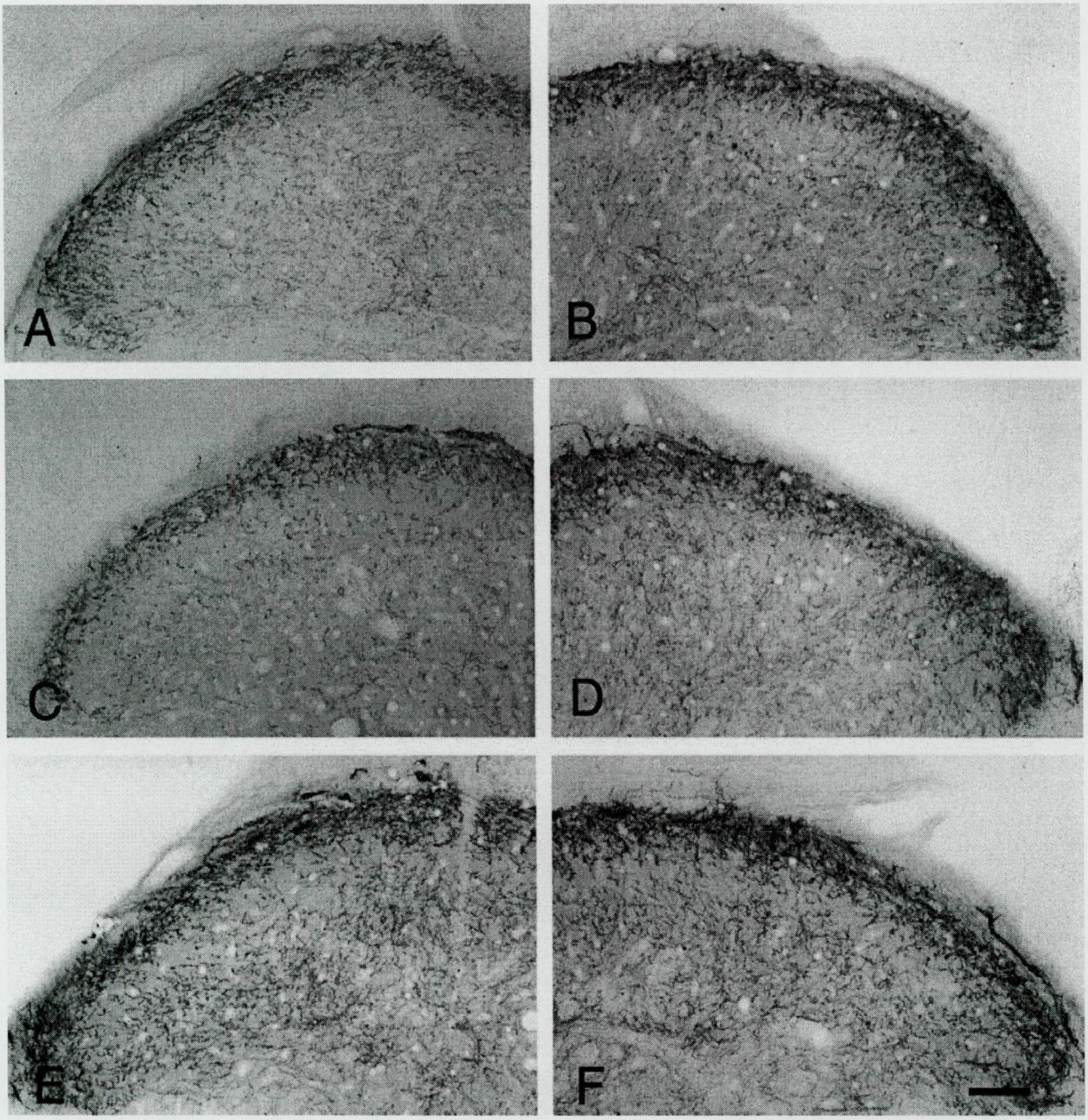

FIG. 5. 5-HT immunoreactivity on transverse sections of the upper cervical spinal cord in (A and B) males, (C and D) ovx and (E and F) ovx + E_2 females. (B, D and F) NTG administration compared to (A, C and E) vehicle increases the immunoreactivity in males and ovx females (B and D) but not in (F) ovx + E_2 rats where the baseline immunoreactivity is higher. Scale bar, 50 μm.

Discussion

To our knowledge this study demonstrates for the first time that systemic administration of NTG is able to change CGRP and 5-HT immunoreactivities in laminae I–II of the spinal portion of trigeminal nucleus caudalis. Up to now, NTG-induced c-Fos (Tassorelli *et al.*, 1995) and NOS activations (Pardutz *et al.*, 2000) were reported in the same area of the upper cervical cord. Release or increased turnover of CGRP in spinal dorsal horns of appropriate segments was reported after localized peripheral inflammation (Sluka *et al.*, 1992), capsaicin administration (Garry *et al.*, 2000) or subcutaneous formalin injections (Zhang *et al.*, 1994). The decrease of the area occupied by CGRP-Ir afferents found here is thus probably a consequence of an increased release of CGRP due to the NO-mediated stimulation of trigeminal nociceptive Aδ and C afferents. Morphological changes suggestive of transmitter release were also found in the distal portions of trigeminal CGRP-Ir afferents in dura mater after electrical stimulation of the gasserian ganglion (Knyihar-Csillik *et al.*, 2000). We could also demonstrate a decrease in the size of CGRP-immunoreactive boutons which would support the hypothesis of an

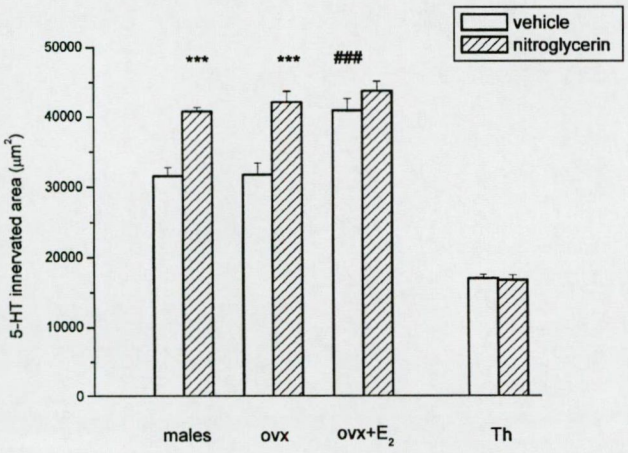

FIG. 6. Histogram showing the area in μm^2 covered by 5-HT-Ir fibres in superficial laminae I–II of sTNC at C1–C2 in the three animal groups and of segment Th1 (th) in males 4 h after subcutaneous injection of vehicle (light bars) or NTG (hatched bars) (mean + SEM, $n = 8$ per group) In males and ovx animals there is a significant increase in the area innervated by 5-HT-Ir fibres (***$P < 0.001$). The level after vehicle administration is significantly higher in ovx + E2 animals than in the male or ovx rats (###$P < 0.001$).

increased release of the peptide due to the NTG administration. By the same token, the NTG-induced increase in 5-HT immunoreactivity in the same spinal grey areas may indicate a reduced release of serotonin from supraspinal afferents. The molecular and functional relationship between 5-HT afferents to the superficial spinal dorsal horn and spinal nociceptors is complex. Multiple 5-HT receptors (such as 5-HT_{1B}, 5-HT_{1D} and 5-HT_{1F}) are found in laminae I–II (Castro *et al.*, 1997). When activated, all of them may decrease c-Fos expression in trigeminal nucleus caudalis induced by nociceptive stimulation (Hoskin *et al.*, 1996; Mitsikostas & Sanchez del Rio 2001). By contrast, spinal nociceptive transmission after peripheral inflammation can be enhanced by the action of serotonin on 5-HT_3 receptors (Green *et al.*, 2000). It is not known whether NTG would reduce 5-HT release in laminae I–II because of a local interaction with serotonergic terminals or via a secondary modulation of the serotonergic descending pain control pathway. Increased 5-HT metabolism was demonstrated in nucleus raphe magnus and in the spinal dorsal horn after subcutaneous injection of formalin (Puig *et al.*, 1992). Carrageenan-induced inflammation in the paw also increased the 5-HT concentration in periaqueductal grey and the lumbar spinal dorsal horn of the rat (Zhang *et al.*, 2000). Whatever the mechanism might be, it seems to be selective for spinal trigeminal nucleus caudalis, as we found no detectable change in superficial dorsal horn of the thoracic spinal cord, a selectivity already pointed out for NTG-induced NOS activation in second-order nociceptors (Pardutz *et al.*, 2000). The selectivity of the NTG effect for the projection site of trigeminovascular afferents underlines the relevance of the NTG model for migraine. The reason for this selectivity has to be determined, but it might be related to the presence on visceral trigeminal Aδ and C afferents of specific receptor populations, such as the 5-$HT_{1B/D}$ receptors, or to other regional differences in neuronal metabolism. Interestingly, the specific 5-$HT_{1B/D}$ agonist eletriptan, which is highly effective in migraine treatment, prevents the morphological changes of CGRP-Ir peripheral dural afferents induced by trigeminal ganglion stimulation (Knyihar-Csillik *et al.*, 2000).

Contrary to the acute changes in immunoreactivities occurring after NTG injection, those found after the experimental modulation of estradiol levels are on a different time scale and reflect slower plastic adaptations of neurotransmitter expression. Our results suggest that ovarian hormones, which greatly influence the course of migraine, have indeed the capacity to modify the expression of pivotal transmitters in the trigeminovascular nociceptive pathway. In animals with high estradiol levels, i.e. ovariectomized females treated with 17β-estradiol, CGRP innervation was clearly reduced in the superficial sTNC layers, compared to animals with low estradiol levels, i.e. males and ovariectomized nontreated females. This is in line with findings by Moussaoul *et al.* (1996). Estrogen receptors are present on spinal sensory ganglion neurons (Yang *et al.*, 1998; Taleghany *et al.*, 1999) and in spinal grey matter (Shughrue *et al.*, 1997). Estradiol may thus act at the genomic level, which would modify the phenotypic expression of CGRP and annihilate any detectable change in immunoreactivity after NTG.

Contrasting with CGRP, the area covered by 5-HT-Ir fibres in sTNC laminae I–II was higher in estradiol-treated ovariectomized rats. This finding has to be discussed in the light of the well-documented effect of estrogen on various aspects of serotonin transmission and metabolism in other brain areas. It has been shown that ovarian steroids increase tryptophan hydroxylase in species such as macaques or guinea pigs (Pecins-Thompson *et al.*, 1996; Lu *et al.*, 1999; Bethea *et al.*, 2000), whilst they decrease mRNA expression of the serotonin re-uptake transporter in monkeys and rats (Pecins-Thompson *et al.*, 1998; Rehavi *et al.*, 1998). Estrogen is also able to desensitize 5-HT_{1A} receptors in hypothalamic neurons (Raap *et al.*, 2000), which may activate serotonergic neurons by decreasing auto-inhibition. Among these various and possibly interrelated effects, an estrogen-dependent increase in activity of the synthesizing enzyme tryptophan hydroxylase in descending raphe–spinal serotonergic neurons would probably best account for our finding of increased 5-HT immunoreactivity in sTNC after estradiol treatment. Estrogen receptors are known to be localized on serotonergic neurons in raphe nuclei (Leranth *et al.*, 1999) and may mediate some of these effects. Like for CGRP, there was no significant change in the sTNC 5-HT innervation after NTG in estradiol-treated ovariectomized rats, probably because there was no possibility for further 5-HT increase after stimulation of its anabolism by the estrogen. On the other hand, it has been shown that estradiol induces structural synaptic remodelling in certain sex steroid-responsive brain areas (Garcia-Segura *et al.*, 1994), so that the changes in the CGRP- and 5-HT-Ir innervation pattern observed here in estradiol-treated animals could be the result of synaptic rearrangements in the superficial laminae of trigeminal nucleus caudalis. Whether they may be relevant for the hormonal influences in migraine remains speculative. It is interesting, however, to draw a parallel between our immunocytochemical data in rats showing that estradiol is able to suppress activation of trigeminal afferents by nitroglycerin, a well-known trigger of migraine attacks (Olesen *et al.*, 1993), and the clinical observation that migraine markedly improves in most women during pregnancy, i.e. when sex hormone levels are high and stable (Marcus, 1995; Silberstein & Merriam, 2000).

In summary, NTG, a NO donor, is able to decrease the area covered by peripheral CGRP-Ir afferents in superficial laminae of rat spinal trigeminal nucleus caudalis and to decrease the area occupied in the same laminae by descending 5-HT-Ir afferents. These effects are annihilated in animals in which the baseline activity of CGRP is decreased or that of 5-HT is increased because of chronically high levels of estradiol. Whatever the underlying molecular mechanisms of the acute NTG-induced and the chronic estradiol-dependent change might be, both seem to be selective for the trigeminal sytem and may shed some light on the role of NO donors and ovarian steroids in trigeminovascular pain syndromes, such as migraine. If, as deduced from our immunocytochemical observations, the release of CGRP is

indeed enhanced and that of 5-HT reduced by NO at the level of the spinal trigeminal nucleus, one may expect increased nociception. On the other hand, the opposite changes in animals with high estradiol levels would lead to decreased trigeminal nociceptive transmission. This might be at least part of the neurobiological explanation for the attack-triggering effect of NTG and other NO donors in migraine patients and for the protective action on this disorder of high and steady levels of ovarian hormones, as for instance during pregnancy.

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Abbreviations

5-HT, serotonin; CGRP, calcitonin gene-related peptide; Ir, immunoreactive; NO, nitric oxide; NOS, nitric oxide synthase; NTG, nitroglycerin; ovx, ovarectomized female rats; ovx + E_2, ovarectomized female rats treated with estradiol; sTNC, spinal C1–C2 portion of trigeminal nucleus caudalis.

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III.

Nitroglycerin-induced nNOS increase in rat trigeminal nucleus caudalis is inhibited by systemic administration of lysine acetylsalicylate but not of sumatriptan

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Systemic administration of nitroglycerin (NTG), a nitric oxide (NO) donor, in migraineurs triggers after several hours an attack of which the precise mechanisms are unknown. We found previously in rats that nitroglycerin (10 mg/kg s.c.) is able to increase significantly after 4 h the number of neuronal nitric oxide synthase (nNOS)-immunoreactive neurones in the cervical part of trigeminal nucleus caudalis. In the present experiments, we demonstrate that the 5-$HT1_{B/D}$ agonist sumatriptan (0.6 mg/kg s.c.) does not alter this phenomenon when given before NTG. By contrast, pretreatment with lysine acetylsalicylate (50 mg/kg i.m.) attenuates the NTG-induced nNOS expression in the superficial laminae of trigeminal nucleus caudalis. These findings suggest that effect of NTG on nNOS at a high dosage may involve the cycloxygenase pathway and that activation of the peripheral 5-$HT1_{B/D}$ receptors is not able to modify this effect. These data could help to better understand the role of NO in the pathogenesis of headaches and the action of antimigraine drugs. □*Nitroglycerin, nitric oxide synthase, caudal spinal trigeminal nucleus, sumatriptan, lysine acetylsalicylate*

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Introduction

Systemic administration of nitroglycerin (NTG), a nitric oxide (NO) donor, in migraineurs can trigger attacks without aura after a delay of several hours (1, 2). Like spontaneous migraine attacks, the NTG-induced attacks can be interrupted by the 5-$HT1_{B/D}$ agonist sumatriptan (3), but their precise neurobiological mechanisms are still unknown. NTG was recently reported to induce a also delayed tension-type headache in patients suffering from chronic tension-type headache (4).

There is no doubt that NO and neuronal nitric oxide synthase (nNOS) play an important role in the processing of nociceptive information, and especially in sensitization of spinal second order nociceptors (5, 6). nNOS is present in the trigeminal system which suggests that it is similarly involved in the processing of cephalic pain (7). NOS inhibitors reduce c-fos activation by nociceptive stimuli in rat dorsal horn (8, 9) and can antagonize neurogenic activation of the trigeminovascular sytem (10). According to preliminary studies, they are also able to reduce pain in migraine attacks (11) and chronic tension-type headache (12).

NTG increases c-fos (13) and nNOS (14) expression in the spinal portion of trigeminal nucleus caudalis (TNC) in rats. In the latter study we hypoth-

esized that the increased nNOS expression in second order trigeminal nociceptors might initiate a self-amplifying process of NO production at the basis of central sensitization. The increased nNOS expression could be due to a direct effect of NO on TNC neurones, but, because of its regional selectivity, it is more likely secondary to activation of primary trigeminal afferents. 5-HT_{1D} receptors are located presynaptically on the distal and proximal portions of these afferents (15, 16) and their activation is thought to contribute to the antimigraine effect of 5-$HT1_{B/D}$ agonists, also called triptans (17). Non-steroidal anti-inflammatory drugs (NSAIDs), such as acetylsalicylic acid (Aspirin), are also effective in the treatment of acute migraine headache (18) and tension-type headache (19). This effect could to be due to their inhibitory action on cyclo-oxygenase 2 (COX-2) and prostaglandins in the spinal trigeminal complex (20), but they could also inhibit NOS activation by reducing the induction of transcription factor NF kappa β (21). Concordantly, it was shown that pretreatment with indomethacin reduces the NTG induced c-fos activation in the caudal trigeminal nucleus (22).

The aim of the present study was to determine if NTG-induced nNOS expression in rat trigeminal nucleus caudalis, as shown in our previous study (14), is altered by pretreatment with the 5-$HT_{1B/D}$ agonist sumatriptan or with lysine-acetylsalicylate (lys-ASA), a NSAID, both of which are effective in the acute treatment of migraine attacks (23).

Methods

The procedures of this study followed the guidelines of the International Association for the study of Pain and the European Communities Council (86/609/ECC). They were approved by the Ethics Comittee of the Faculty of Medicine, University of Liège. Seventy-eight male Wistar rats (250–350 g) were used. For immunohistochemistry, 48 animals were divided into three groups of 16. In the first group the rats did not receive any pretreatment. In the second group the rats received a subcutaneous (s.c.) injection of sumatriptan (Imitrex, Glaxo Smith Kline) at a dose of 0.6 mg/kg. The animals of the third group were injected intramuscularly with lys-ASA (Aspegic, Sanofi-Synthelabo) at a dose of 50 mg/kg. Sumatriptan was diluted in physiological saline, lys-ASA in its commercially available solvent (distilled water). Ten minutes later in each group 8 animals received a s.c. injection of NTG (prepared from Nitrolingual pumpspray, Pohl-Boskamp GmbH, Germany) at a dose of 10 mg/kg and 8 animals received a s.c. injection of the vehicle (gift from Pohl-Boskamp).

Four hours after the NTG or placebo injections the rats were deeply anaesthetized by pentobarbital (Nembutal 80 mg/kg) and transcardially perfused with 100 ml physiological saline followed by 500 ml 4% paraformaldehyde in phosphate-buffered saline (PBS). The portions of cervical spinal cords comprised between –5 and –11 mm from the obex were removed and postfixed overnight for immunohistochemistry.

For Western blotting 30 rats were divided in three groups of 10 and received pretreatment and NTG or placebo injections as above. Four hours later, they were deeply anaesthetized and transcardially perfused with 200 ml of physiological saline. Cervical spinal cords between –5 and –11 mm from the obex were removed and frozen in liquid nitrogen for Western blotting.

Immunohistochemistry

After cryoprotection (30% sucrose overnight) 30 µm thick cryostat sections were cut and serially collected in 16 wells containing cold PBS. Each well received sections at a 0.5-mm distance throughout the rostro-caudal extent of the C1-C2 spinal cord. After pretreatment with 0.3% H_2O_2, the free-floating sections were rinsed several times in 0.1 M PBS containing 1% Triton X-100 and then kept for 2 nights at 4°C in polyclonal antinNOS antibody (Euro-Diagnostica, B 220–1) at a dilution of 1 : 20000. The immunocytochemical reaction was visualized using the avidin-biotin kit (ABC) of Vectastain (Vector Laboratories Inc., PK-6101) and staining with nickel-ammonium-sulphate-intensified 3′,3′-diaminobenzidine. Specificity of the immune reactions was controlled by omitting the primary antiserum.

Western blotting

The dorsal portions of spinal cord segments were homogenized in cold Tris-HCl buffer (50 mM, pH = 7.4). Protein concentration was measured according to Bradford (24) using BSA as a standard. Equal amounts of protein samples (20 µg/lane) were separated by standard SDS-PAGE procedures at 200 V for 1 h and transferred to immobilon P membrane (Millipore). Following the transfer and blocking in 5% nonfat dry milk, membranes were incubated with a nNOS antibody (Transduction Laboratories, N31020), diluted to 1 : 1000. After the detection of nNOS protein the membranes were stripped and reprobed for β-actin antibody (Sigma A5441) diluted

to 1 : 4000, which was used as an internal control. Protein bands were visualized using the ECL Western blotting analysis kit (Amersham). They were quantitatively analysed using a laser densitometer (Pharmacia LKB). Optical densities of specific bands were quantified by densitometry and corrected for protein loading by dividing by the β-actin signal of the same sample.

Statistical analysis

nNOS-positive cells were counted by an observer blinded to the procedures in laminae I-III of the cervical spinal cord, in three different series of sections in each animal. The individual sections in these series were taken at 0.5 mm distances along the rostrocaudal axis. nNOS-positive neurones were scored, if they contained cytoplasmic and dendritic staining and a nucleus. The cell counts of nNOS and of relative Western blot optical densities were analysed with multiple variance analysis (ANOVA) and post hoc test Scheffe. The statistical tests were performed by StatWiew (Version 4.57 for Windows, Abacus Concepts Inc., Berkley, NC, USA). Significance level was set at $P < 0.05$.

Results

Transverse sections of the cervical spinal cord demonstrated numerous nNOS-IR neurones in dorsal horn laminae I-III. These cells are small to medium sized neurones (8–15 µm diameter) with few dendrites were most common. There was no significant difference in the number of NOS positive cells at different levels of the C1-C2 region. NTG induced an increase of the nNOS-IR cells in the superficial layers of the caudal trigeminal nucleus in control, non-treated rats. A similar increase in the number of immunoreactive neurones was observed in the sumatriptan-pretreated group of animals. Lys-ASA pretreatment, on the contrary, abolished the NTG effect on nNOS-positive neurones (Figs 1 and 2).

Western blot analysis of the C1-C2 region confirmed the results obtained by immunohistochemistry. We could identify a band at 155 kD characteristic for the nNOS protein. In animals, which had received NTG 4 h before, the density of the C1-C2 nNOS protein band was increased compared to vehicle-injected rats. This increase was similar in control and sumatriptan pretreated groups. After pretreatment with lys-ASA, the nNOS band was comparable after NTG or vehicle injection (Fig. 3). Densitometric analyses of the bands (corrected for protein loading) confirmed the results of the visual inspection of the Western blots: relative to vehicle injections, the optical density of the nNOS band was significantly enhanced after NTG administration with or without sumatriptan pretreatment, but not when the NTG injection was preceded by a pretreatment with lys-ASA (Fig. 4).

Discussion

To the best of our knowledge, this is the first study, which examines the effect of antimigraine drugs on the previously described NTG-induced increase of nNOS expression in the superficial layers of trigeminal nucleus caudalis (14). Pretreatment with sumatriptan failed to influence the increase in nNOS expression induced by the NO donor NTG, while the NSAID lys-ASA attenuated the NTG-induced nNOS activation.

It is not known how NTG modifies nNOS in TNC. There are several possible ways for NO to induce nNOS expression in secondary trigeminal nociceptors:

- a direct effect on these neurones;
- via local laminae I-II interneurones;
- via peripheral nociceptive afferents to TNC;
- via descending central pathways.

Although in our previous study (14) the NTG effect on nNOS seemed to be specific for TNC as it was not found in upper thoracic segments, we cannot rule out the involvement of other neuronal subpopulations. It has been shown indeed that NO can exert a dual effect on nociception depending on the dose used (25, 26). Moreover, the exact extent of nNOS enhancement in TNC has not been determined; it may not be exclusively related to nociception and may vary between species. We hypothesized nonetheless from our first study that the most likely explanation was a secondary activation of second order nociceptive neurones and/or interneurones because of excitation by NO of their peripheral afferents (14).

If the latter hypothesis is correct, one would expect that an agonist of presynaptic 5-$HT_{1B/D}$ receptors which inhibit the release of transmitters in primary nociceptive afferents (27), is able to attenuate the NO effect on nNOS expression. Sumatriptan was indeed shown to reduce the CGRP release from meningeal trigeminal fibres after electric stimulation of the Gasserian ganglion in the rat, while lys-ASA was not (28). It was also reported that sumatriptan prevents the increase of dural perivascular CGRP-IR nerve endings after electric Gasserian ganglion stimulation (29). The

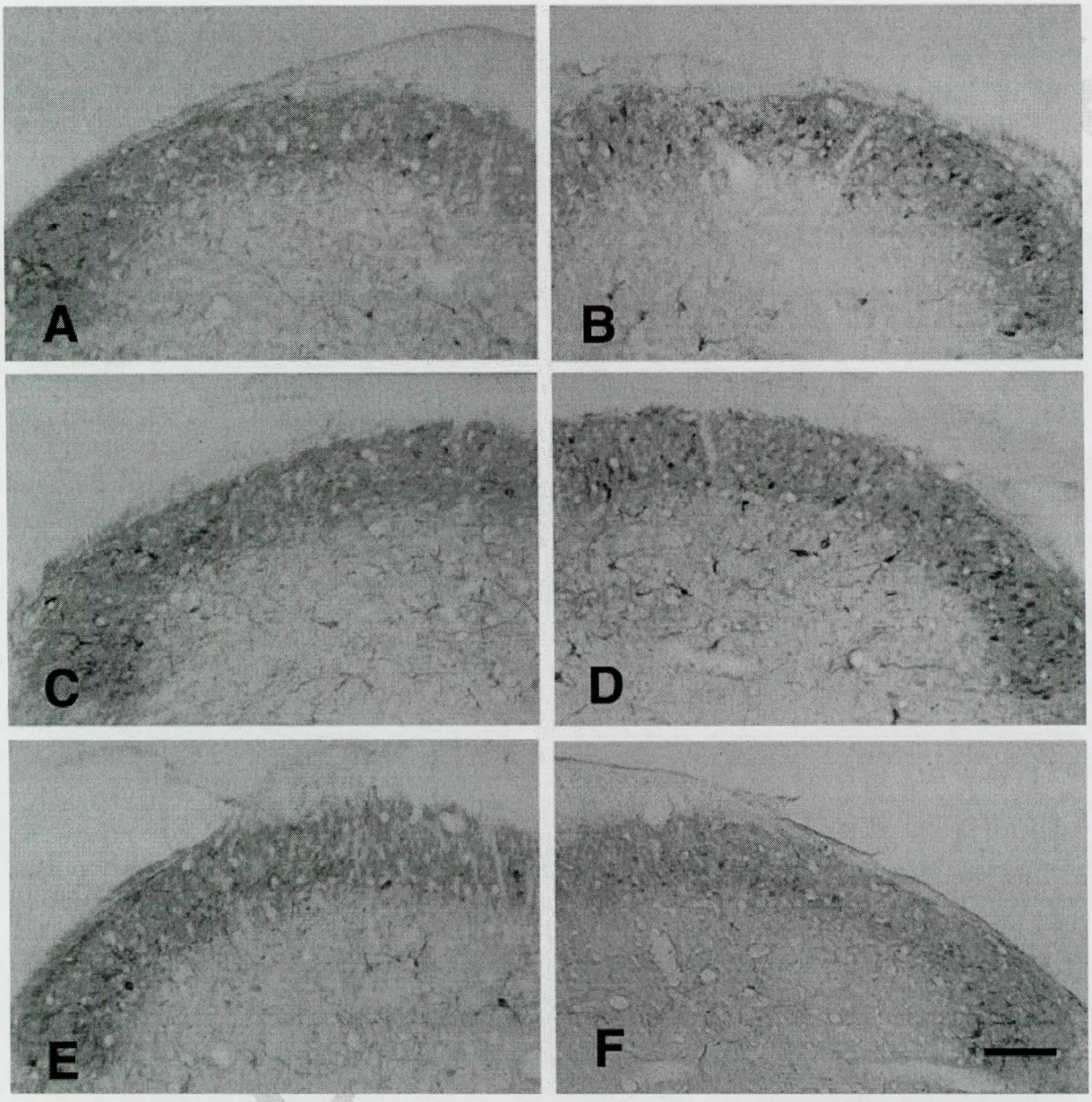

Figure 1 nNOS-immunoreactivity on transverse sections of the upper cervical spinal cord, at –6 mm from the obex, in non pretreated (control) (A,B), sumatriptan-pretreated (C,D) and lysine-acetylsalicylate-pretreated (E,F) rats. Compared to vehicle (A,C,E), subcutaneous NTG administration (B,D,F) increases the number of nNOS-immunoreactive cells in controls (B) and after sumatriptan pretreatment (D) but not after lysine-acetylsalicylate-pretreatment (F). Scale bar = 50 μm.

lack of effect of sumatriptan in our study may therefore suggest that activation of nociceptive afferents has no role in the NTG-induced nNOS increase or that 5-$HT1_{B/D}$ receptors are not capable to oppose such a chemical activation. Earlier studies on migraine headache have emphasized a possible role for 5-$HT_{2B/C}$ (30), or more likely for 5-HT_{2B} receptors, which mediate the release of NO from the endothelium (31). Recent experiments showed that 5-HT_{2A} receptor activation was able to enhance nNOS expression in trigeminovascular neurones (32) and up-regulation of platelet 5-HT_2 receptors was reported by the same group in transformed migraine (33). Taken together these findings underline that, besides 5-$HT_{1B/D}$, other 5-HT receptor subtypes play a role in trigeminovascular

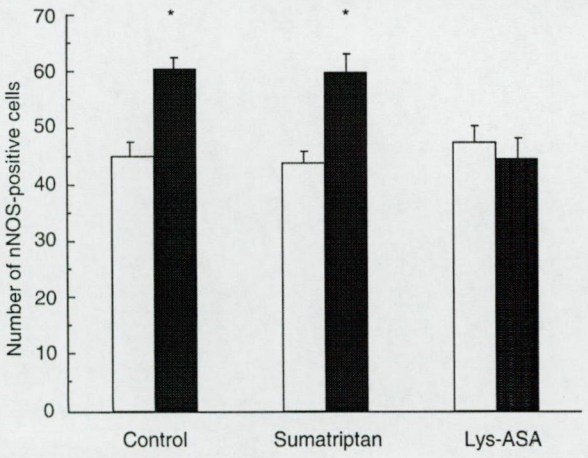

Figure 2 Histogram showing the mean number of nNOS-immunoreactive cells in the superficial layers of the C1-C2 dorsal horns in the 3 animal groups 4 h after subcutaneous injection of vehicle (□) or NTG (■) (mean + S.E.M, $n = 8$ per group). There is a significant increase of nNOS-immunoreactive cells after NTG injection in control and sumatriptan-pretreated rats ($^*P < 0.05$), but not in lysine-acetylsalicylate pretreated rats.

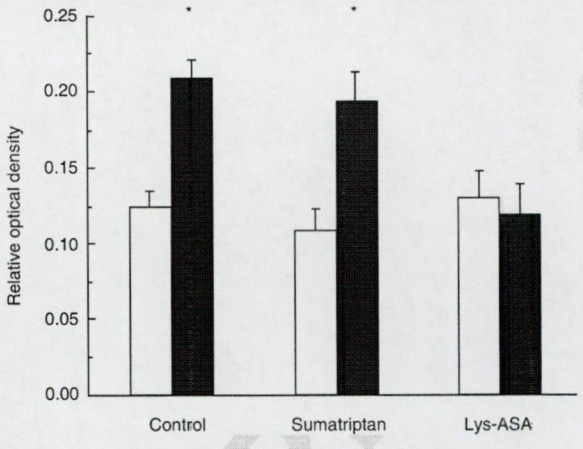

Figure 4 Histogram showing the optical densities of nNOS Western blots in the C1-C2 segments of the 3 animal groups 4 h after subcutaneous injection of vehicle (□) or NTG (■) (mean + S.E.M, $n = 5$ per group). Data are expressed as proportion of β-actin. In controls and after sumatriptan pretreatment there is a significant increase of optical density in the 155 kD nNOS band ($^*P < 0.05$), while this increase is absent after lysine-acetylsalicylate pretreatment.

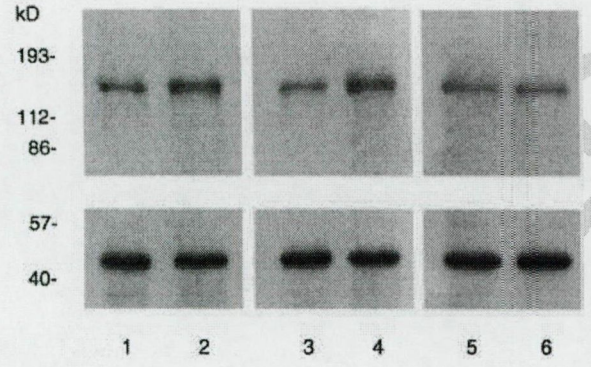

Figure 3 Western blotting of nNOS in C1-C2 spinal cord in control (lanes 1,2), sumatriptan- (lanes 3,4) and lysine-acetylsalicylate pretreated (lanes 5,6) animals. Compared to the vehicle (1, 3, 5), NTG administration (2, 4, 6) enhances the nNOS band (155 kD) in the control (2) and sumatriptan-pretreated group (4), but not in the lysine-acetylsalicylate-pretreated group (6). The corresponding β-actin bands are shown below for each animal group.

pain, which could be an explanation for the lack of effect of sumatriptan in our model.

An alternative explanation for the ineffectiveness of sumatriptan must be taken into account: its hydrophilicity and reduced ability to cross the blood–brain barrier (34). For instance, in cat systemic administration of sumatriptan is able to inhibit the firing of central trigeminal neurones induced by sagittal sinus stimulation only after disruption of the blood–brain barrier by mannitol (35), which contrasts with more lipophilic triptans like zolmitriptan and naratriptan (36, 37). Interestingly, in acute migraine treatment sumatriptan has little efficacy, if it is taken at the time of the aura, i.e. before the headache phase (38), whereas aspirin will not lose efficacy on the headache if given during the aura (39).

There is no doubt that sumatriptan is effective in NTG-induced attacks in migraineurs (3), but such attacks are triggered by microgram-range doses of NTG administration, which seems to produce sensitization, but not c-Fos activation of trigeminal neurones (40). It may thus be possible that higher doses of NTG, such as those used here, induce a more robust activation of central trigeminal nociceptors.

Inducible cyclo-oxygenase 2 (COX-2) is expressed in the superficial dorsal horn of the rat spinal cord (41) and plays a role in central sensitization (42). It mediates most of the analgesic effects of NSAIDs (20). After hindpaw inflammation in mice some of the COX-2 expressing cells in laminae I-II are also positive for nNOS (43) suggesting an interaction between the two enzymes. Lys-ASA, like all NSAIDs, inhibits COX-1 and COX-2. It is effective in migraine, penetrates easily the blood–brain barrier and has a long-lasting effect as well as a long half-life (44). Lys-ASA may also exert an antinociceptive effect by acting directly upon the periaqueductal grey matter (45). Taken together, these data and our

present results suggest that the stimulating effect of NTG, and thus of NO, on nNOS expression in secondary trigeminal nociceptors might be mediated by prostanoids and in particular by COX-2 expressing interneurones in TNC superficial laminae. To prove this hypothesis, further studies exploring the NTG effect on spinal COX-2 and the relative time course of nNOS and COX-2 expressions in the trigeminal sensory complex, are necessary.

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